AFM automated methodology to rapidly analyze hundreds of cells.

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Recommendations for maximizing the number of wells analyzed.

To successfully implement our methodology in any JPK system some recommendations must be follow:

1. Reduce the tilt of your stamp. Align in the best possible way (by sight) your stamp, Figure 1A shows a recommendation about the way the stamp should be placed to successfully work.
2. Select the initial points. In a force map image or QI image, select as the initial well W1 the one with a positive value on X and a positive value on Y (+, +). And for W2 select a well which central coordinate has a positive value on X and a negative value on Y (+, -). Figure 1B shows the recommended wells to select related to the centering algorithm, this is because the algorithm creates a grid that covers the surroundings of W1 (Figure 3A). If W1 is too close to the edge of the scanning area, then the grid would not be created and the program will throw an exception.
3. Square distribution. The program assumes distances between the rows and columns of the patterns are the same (for X and Y) this means that if your pattern has a different distance for rows and columns it will not find the other cells.
4. Placing the stamp on the stage. Usually the measurements on cells are done in liquid media, because of this we recommend to place the stamp inside a petri-dish so the cells will be in liquid the whole time. In our case the PDMS stamp have good adhesion to the petri-dish.

Figure S1. Execution Recommendations. A: Optical images of the PDMS stamp recommended tilt. B: Position of the cantilever related to the initial well for a guarantee execution of the centering algorithm. Black square represents the maximum dimensions of a scanning area; the red dots represent wells close to the edge of the area that make impossible the execution of the centering algorithm. Green dots represent a good selection of a well that is near the edge of the scanning area.
**Algorithm for automated measurements**

The flowchart for the algorithm is shown on Figure 2A. The first steps consist in acquiring the input parameters: two initial central coordinates (wells W1 and W2, Figure 2C), manually selected; the size of W1, the pitch between the well, and the size of the total area to be scanned (400 µm). The script calculates the scanning areas coordinates by using the Δ distance (distance between W1 and W2) plus half the size of W1. With those parameters the algorithm divides the maximum area into scanning areas (Step 2, Figure 2B), and the centering algorithm is executed (Step 3, see centering algorithm), to find the true center of the initial wells (W1’ and W2’). With this information the positions of all the wells inside the scanning area and the tilt angle can be calculated accurately.

Then, the cantilever is moved toward each microwell and inside each microwell, several nanoindentations are performed guaranteeing measurements on different regions of the cells (Step 4). To perform the nanoindentations inside the microwells a safe area was defined (a confined square of 1,5 µm x 1,5 µm, see force curve analysis section) to avoid measures at the microwell edge or on PDMS.

Once all the microwells of the scanning area have been measured the piezo is retracted, then the cantilever goes back to W1’ and the motor stage activates and moves the sample to the next 100x100 µm² scanning area (Step 5). However, the motor stage creep prevents the cantilever to be exactly at the center of the new W1 microwell of this new 100x100 µm² scanning area, to correct this the centering algorithm is executed again. Nevertheless, the centering is executed only once, because now the tilt angle is known and it does not change in between scanning areas. The automated process described in the last paragraph can be seen in the supp. video.
Figure S2. Initializing AFM automatic acquisition. A: Flowchart of the algorithm developed for automated measurements with the AFM. B: Optical image of the patterns after the cell immobilization process, the numbered squares represent the scanning areas. C: AFM topographical image recorded in force-volume mode. W1 and W2 are the initial microwells used to measure the distance $\Delta$ needed to divide the pattern into scanning areas. Blue lines represent the alignment axis of the piezo and the green lines are the axis of the pattern. $\Theta$ is the tilt angle between those two axes.
Centering algorithm.

To find the center of a well the centering algorithm first creates a grid of 25 coordinates distributed in an area of 7.5 µm x 8.5 µm (Figure 3A), then the tip is approach to the first coordinate until is in contact with the surface (Figure 3B), once this is done the height is stored (Figure 3B, red dotted line) and the tip is retracted. The procedure is repeated for every coordinate in the grid. The coordinates marked C1, C2 and C3 are used for plane calculation, the plane is used to know the relative height of the flat PDMS surface. The plane equation is defined as Ax+By+Cz +D = 0 so is necessary to find the coefficients A, B, C, and D, to do this the following equations are used.

\[
A = (C_{3,y} - C_{1,y})(C_{2,z} - C_{1,z}) - (C_{2,y} - C_{1,y})(C_{3,z} - C_{1,z}) \\
B = (C_{3,z} - C_{1,z})(C_{2,x} - C_{1,x}) - (C_{2,z} - C_{1,z})(C_{3,x} - C_{1,x}) \\
C = (C_{3,x} - C_{1,x})(C_{2,y} - C_{1,y}) - (C_{2,x} - C_{1,x})(C_{3,y} - C_{1,y}) \\
D = -(A)(C_{1,x}) + B(C_{1,y}) + C(C_{1,z})
\]

Then the distance \(d\) between each of the 25 points \((x,y,z)\) and the stamp plane is calculated thanks to equation V.

\[
d = \frac{Ax + By + Cz + D}{\sqrt{A^2 + B^2 + C^2}}
\]

And finally this distance is used to determine which points fall inside the wells and which are on the flat PDMS surface, this is done by assigning weights to each point, the points near or on the surface gets a zero weight, the points inside the well receive a weight>1. The points with zero weight are discarded and only points with weight>1 (Figure 3B, green dots) are used in equation VI to find the centroid of a polygon, which is thus considered as the more precise center of the well.

\[
Centroid = \frac{\sum_{i=0}^{\max} z_i(x_i,y_i)}{\sum_{i=0}^{\max} z_i}
\]

Figure S3. Centering algorithm. A: 3D schematic of a well and the grid of 25 points, C1, C2 and C3 are the points used for plane calculation. B: schematic representation of the approaching of the tip to the well on different regions, the blue asterisks represent the points that fall on the PDMS surface and that will be used for plane calculation, the green asterisks represent the points used to find the center of the well, the green line represents the baseline of the piezo.
**Force curves acquisition and analysis**

The massive acquisition of force curves done with our methodology forced us to guaranty certain level of reliability on the measurement procedure, so it’s important to take into consideration the respective tip and microwell geometries. Figure 4A shows the square microwells where the cells are immobilized. The microwell can be divided into two areas: i) the safe area (green) where the tip does not touch the walls of the microwells (calculated considering the dimensions of the tip). Force curves are exclusively recorded in the area which is also the region of the cell (Figure 2B). And ii) the edge (red) where the tip interacts with the walls before touching the cells leading to erroneous measures. This area is avoided and no measurements are performed there.

The cells stiffness was extracted from the force curves, using the JPK data processing software, which performs a linear fit on the retract curve as can be seen in Figure 4C. In this figure, the difference between treated and native cells is observed. PDMS information is used for filtering, because the filling rate of the PDMS stamp is not 100 % and therefore some of the force curves are recorded on PDMS instead of a cell. Based on its stiffness and contact point value (Figure 4D) is possible to discard curves recorded on PDMS.

![Force curves acquisition and analysis](image)

Figure S4. Acquisition method. A: schematic of the area inside the microwells where the measurements are performed, in the red area the tip is crushing against the walls of the microwells on the contrary in the green area the tip is reaching the bottom of the microwell where the cells are immobilized, α is the semi-angle of the tip (17.5°). B: shows the microwell schematics including an example of an immobilized cell and how is expected to be distributed in the red and green areas. C: Force-Height curves (retract segment) of the different cells analyzed, the linear fit shows the segment of the curve used to extract the spring constant value. D: Approaching force curve showing the contact point and its relation with the depth of the microwell.
Spring constant and cell population heterogeneity analysis.

To obtain a better idea of the cells values inside the cell population we took each well that contained a cell (by applying the filtering process to the force curves), and obtain the median of those force curves so in the end only one value will represent the cell trapped on the well. Figure 5A and 5B shows the histograms of the spring constant for native cells (experiment 1) and cells treated with caspofungin (experiment 3) respectively. On these histograms is possible to see the presence of two peaks and their shift to the left after the treatment. Figure 5C and 5D shows the histograms for native cells (experiment 2) and treated (experiment 4). When the median values per cell are plotted is more evident the presence of the two peaks.

Figure S5. Histograms of the median spring constant values. A and B: Show the median results per cell for native and caspofungin treated cells, experiments 1 and 3. C and D: Show the median results per cell for native and caspofungin treated cells, experiments 2 and 4.
Time-position dependency

The histograms in Figure 6A and 6B show the values of Spring constant as time evolves, it can be seen that the contributions from the peaks are present at every hour which help discard the idea of the peaks are present because of the time it took to perform the experiment. Figure 6C and 6D show four points of indentation (central points of the cell) for experiments 1 and 3 and nine points of indentation for experiments 2 and 4 related to the spring constant values; if the presence of peaks were to be related to the position of indentation, the plot will show specific contributions according to a position.

Figure S6. Time-position dependency of values. Histograms in the center are the original data which is divided into the different subgroups corresponding to the subpopulations founded (cyan/green). A and B: Show the presence of the two sub-populations at every hour in the experiment. C and D: show the positions of indentation; on each position it is possible to see the presence of the subpopulations (cyan/green). Subgroup organization was done using the k-means algorithm.